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Nanoparticles loaded with garden cress (*Lepidium sativum L.*) extract induce apoptosis in breast cancer

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ABSTRACT

Garden cress herb (*Lepidium sativum L.*) extract has recently gained attention in research over the past years. Many of the published research articles discuss are its use in treating different types of cancer. Nanoparticles are a modern research tool that helps with targeting the drugs towards the cancerous cells while evading the healthy ones depending on properties that are present in cancer cells but not in normal ones. In this article, the authors examined the effect of garden cress (*Lepidium sativum L.*) extract on breast cancer cell lines (MCF-7 and MDA-MB-231) with and without being loaded onto nanoparticles. The viability of the cells was measured at the beginning, after treatment with the extract alone, and after treatment with the extract-loaded nanoparticles. Wound healing assay results showed better effect of the nanoparticles loaded with the extract. The entrapment efficiency of the nanoparticles was calculated based on electron microscopy results. Our team used the FACS assay to measure cell death by either apoptosis or necrosis. Detection of necrotic and apoptotic cell deathwas carried out by using Annexin V and propidium iodide (PI) staining and analyzed using flow cytometry. Results showed increased induction of apoptosis in cells treated with nanoparticles loaded with *Lepidium sativum L.* extract versus cells treated with the extract alone.

Keywords: *Lepidium sativum* L., nanoparticles, breast cancer, electron microscopy, zeta potential.

1. INTRODUCTION

Cancer is among the top diseases that causes death. In 2018, nearly 10 million people died because of cancer. Over 18 million people were diagnosed with some form of cancer. Breast cancer is the most common type of cancer that affects females (Ferlay et al., 2019). Breast cancer is a highly metastatic cancer that firstly affects nearby lymph nodes under the arm and then proceeds to metastasize to other tissues, most commonly the lungs, bones, and the liver (Scully et al., 2012). There is an elevation in the number of research articles involving the use of the extract of *Lepidium sativum* in the treatment of many diseases (Alqahtani et al., 2019). Garden cress (belonging to family

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Brassicaceae) extract is characterized by its rich content of minerals, amino acids, and alkaloids. Alkaloids are a set of heterocyclic chemical compounds that possess a nitrogen atom. *Lepidium sativum* extract possesses many pharmacological activities such as antifungal, antibacterial, and cardiotonic activity (Baregama and Goyal, 2019). Alkaloid extracts are used for their pharmacological activities. The alkaloid activity targeted here is its apoptotic property, where alkaloids interfere with protein and DNA synthesis, thus, obstructing cell growth and division.

L. sativum extract contains derivatives of phytosterols and their parent molecules. These plant phytosterols have anti-inflammatory activity, antioxidant activity. They also play a role in protecting against other illnesses (Moser et al., 2009). Flavonoids are phenolic compounds, which are present abundantly in the extract help fight against oxidative stress that could cause cardiovascular diseases, ageing, or cancer (L'Hadj et al., 2018). A previous research article has shown that the extract of *Lepidium stavium* favors inducing cancer cell death by apoptosis in leukemia in Jurkat E6-1 cells where caspase-3 activity increased with a longer incubation period (Basaiyye et al., 2019). There are many benefits to using nano-particle preparations. The most important advantage is the targeting of the desired loaded molecules to the tumor tissue while evading normal tissues, thus, improving the efficacy of the drugs while reducing the side effects. PLGA-PEG is the most common nanoparticle preparation used for drug-delivery applications. PLGA-PEG is considered to be safe since they are highly biocompatible and biodegradable (Fan et al., 2018).

2. MATERIALS AND METHODS

Duration of the study

Our team carried out the following experimentsover the course of 8 months (Jun 2019-Jan 2020).

Chemicals and Reagents

Poly (D, L-lactide-co-glycolide) (PLGA, copolymer ratio 50:50, Inherent viscosity 0.41) was obtained from (Birmingham polymers, Inc., Birmingham, AL). Polyvinyl alcohol (PVA, MW 30.000), and polyethylene glycol (2 kD) were bought from Sigma-Aldrich Co., Montana, US. Other chemicals used throughout the experiments were of analytical grade.

Preparation of *Lepidium sativum* PLGA-PEG nanoparticles

PLGA-PEGNPs were prepared in solvent evaporation methods using an oil-in-water (O/W) emulsion with some modification of the previously published paper (Abd-Rabou and Ahmed, 2017). Briefly, 50 mgs of PLGA polymer were added to 1.5 ml of chloroform. Thus, a primary emulsion was formed. This primary emulsion was added to aqueous PVA solution (6 ml, 2% w/v) for further emulsification to form an oil-in-water emulsion. A microtip probe sonicator was used for that purpose (VC 505, Vibracell Sonics, Newton, USA). Solution was set over an ice bath, with an energy output of 55W for 2 minutes. There was a continuous overnight stirring of the emulsion for the sake of completely evaporating the organic. Excess amount of PVA was removed the next day by ultracentrifugation at 50, 602 xg under cooling (4 ^{0}C) for 20 min (Sorvall Ultraspeed Centrifuge, Kendro, USA) followed by washing thrice with double distilled water. 5% w/w PEG with molecular weight 2 kD was mixed with the PVA aqueous solution before emulsification to get PLGA-PEG NPs (nano-void). For therapeutic applications, N2 loaded PLGA-PEG NPs(N2N) were prepared similarto the methods previously mentioned, by the addition of 150mg/ml of these compounds to the mixture prior to emulsification.

Characterization of the N2N and N3N

Entrapment efficiency measurement

Dialysis tubing technique was used to purify the synthesized nano-formulations N2N to remove any contaminations and eliminate the non-conjugated free compounds suspended in the mixture by washing it through regenerated cellulose using the same solvent (Amicon 10,000 MWCO ultrafilter, Millipore, USA). The Entrapment efficiency (EE%) for N2N was measured with the BMG Labtech microplate reader (Germany). The ration of the amount of compound incorporated into the NPs to the total amount of compound added was calculated and considered as the entrapment efficiency.

Transmission electron microscopy (TEM)

Particle morphology of the N2N was examined by transmission electron microscopy (TEM, Philips CM-10, FEI Inc., Hillsboro, OR, USA). 100 μg/mL of the nano-suspension were dropped into formvar-coated copper grids, and after the samples dried completely, staining was carried out with uranyl acetate solution (2% w/v) (Electron Microscopy Services, Ft. Washington, PA). Soft Imaging Viewer Software and Digital Micrograph were used to capture the images and analyze them.

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Particle size and analysis of zeta potential

Particle size and analysis of the zeta potential of the NPs were calculated using photon correlation spectroscopy technique (PCS). A Zeta Sizer was used for that purpose (Nano ZS, Malvern Instruments, UK), All samples used in the experiment were kept at temperature of 25.0 ^{o}C.

Cell culture

Normal lung fibroblast cells (WI-38), two breast cancer cell lines (MCF-7 and MDA-MB-231), and two hepatocellular carcinoma cell lines (HePG-2 and HUH-7) were brought from cell culture unit from VACSERA (Cairo, Egypt). The original of the cell lines used is the American Tissue Culture Collection (ATCC). Cells were cultured and propagated in RPMI-1640 medium supplemented with heat inactivated fetal bovine serum (FBS, 10%) and 1% penicillin/streptomycin antibiotic preparation.

Cell viability assay

WI-38, MCF-7, MDA-MB-231, HUH-7, and HePG-2 cancer cells were seeded into 96-well plates (at a density of 5,000 cells/well). On the following day, cells were treated with different concentrations (0, 15.65, 31.25, 62.5, 125, 250, 500, and 1000 μg/mL) of *LS* methanolic crude extract in fresh medium and incubated for another 24 h. Viability of the cells was then measured using MTT assay (Sigma Aldrich). The absorbance was measured at 570 nm. An ELISA microplate reader was used for that purpose (Molecular Devices, Downingtown, PA, USA) (Ismail et al., 2013).

Wound Healing assay

Wound healing is a simple technique used in vitro cancer study to assess metastasis in different cancer cells. Here, we study the effect of *LS* methanolic crude extract on breast cancer cell migration was assessed using the wound healing assay as previously described (Ismail et al., 2013). In brief, when cells reached about 90% confluency, wounds were made manually using a pipette tip. Cells were washed with PBS and then treated for 48 hours with the extract at the indicated doses. The images were then photographed and analyzed with Image J software.

Gene expression analyses

The effect of the different concentrations of *LS* methanolic crude extract (0.00, 200, and 400 μg/mL) on the expression of a panel of selected genes involved in apoptosis in both breast cancer cell lines were examined using real-time quantitative polymerase chain reaction (PCR). Designing the primers for quantitative real-time polymerase chain reaction analyses was carried out using the Primer Express 1.5 software (Applied Biosystems).

The primers were designed as follows: BAX forward; 5′-CCCGAGAGGGTCTTTTTCCGAG-3′ and reverse, 5′-CCAGCCCATGATGGTTCTGAT-3′, BCL-2 forward; 5′- TCAGAGCTTTGAGCAGGTAG-3′ and reverse, 5′-AAGGGCTCTAGGTCATTC-3′, P53 forward; 5′- TGACTGTACCACCATCCACTA-3′ and reverse, 5′-AAACACGCACCTCAAAGC-3′, and GAPDH forward; 5′- TGTCCGTCGTGGATCTGAC -3′ and reverse, 5′- CCTGCTTCACCACCTTCTTG -3′. RNA was extracted. The corresponding cDNA was made, and then the real-time PCR technique was used for gene expression analysis as previously described (Ismail et al., 2013). Data were then analyzed as previously shown in (Livak and Schmittgen, 2001).

Necrotic and apoptotic cell death using FACS analysis

The induced necrotic and apoptotic cell deathin breast cancer cell were measured using propidium iodide (PI) and Annexin V. In brief, MDA-MB-231 and MCF-7 cells were cultured in 10 tissue culture dish with initial number 4 x 10^{5}cell/ mL in RPMI growth media. Plates were treated with (0.0 and 400 μg/mL) of *LS* methanolic crude extract for 48 hrs. Briefly, the cells washed once with PBS and suspended in 100 μL 1X Annexin V binding buffer. 5μL of FITC Annexin V buffer were added and allowed to react for 15 minutes at 25^{o}C. 5 μL of PI buffer were supplemented for every sample. For the last step, 400 μL of 1X Annexin V binding buffer were added to each sample and investigated using Becton Dickinson FACS.

3. RESULTS

Entrapment efficiency

For assessing the value of entrapment efficiency (EE%) of N2 in N2N dialysis tubing technique was performed. The results showed that the EE% was 85% for N2 in N2N.

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Characterization of N2N

We used TEM to illustrate the morphology of the synthesized N2N. Figure 1 A and B showed the TEM image of N2N. The image shows spherical NPs with a nano-capsule of PLGA and PEG.

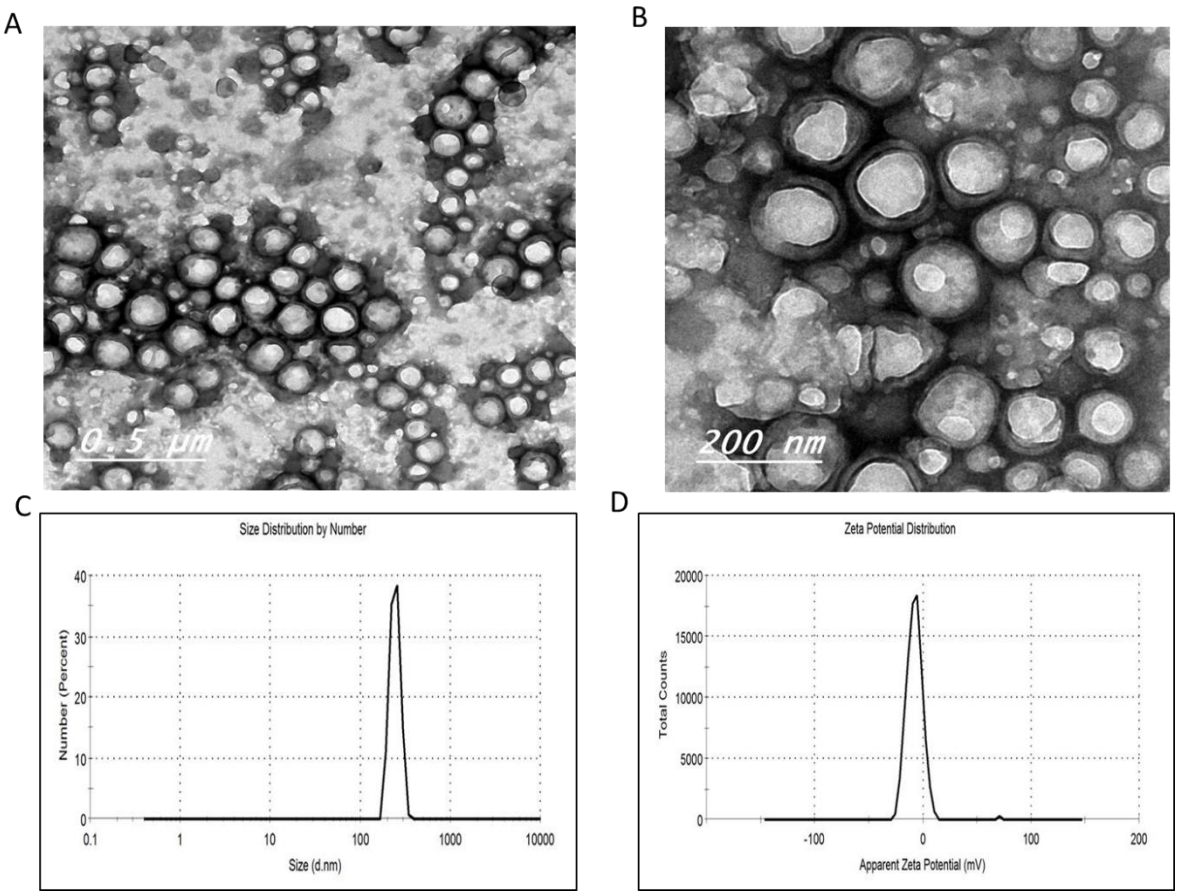

Figure 1 (A&B) TEM image of LsNps. The image shows spherical NPs with a nano-capsule of PLGA and PEG, (C) The size distribution of the LsNps (242 nm) using Zeta Sizer apparatus and (D) The Zeta potential of the LsNps (-7.9 mV) using Zeta Sizer apparatus.

Particle size and zeta potential analyses

N2-loaded PLGA-PEG NPs were characterized by Dynamic Light Scattering (DLS). DSL analysis showed that its Z-average diameter was 242 nm as illustrated in Figures 1C. The polydispersity index (PDI) value was 0.2, which reflects the high stability of the NPs. There was a consistent result of negativezeta potential charge value on the N2-loaded PLGA-PEG (ZP= -7.9 mV) 11 surface of as shown in Figure 1D.

The Nano-formulation was stable; where results of zeta potential value and polydispersity index (PDI) of the synthesized N2N indicated that it is stable formulations. The values of zeta potential peaks as calculated are not near to 0 and all PDI values calculated were smaller than 0.5. These would suffice to bring stabilization to the systems by electrostatic stabilization (Abd-Rabou and Ahmed, 2017).

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Cell Viability Assay

The use of LsNps was able to produce less viable cells (figure 2 A-C) upon treating different cell lines with the preparation. Higher concentration corresponded to less viable cells in the entire different cell lines used.

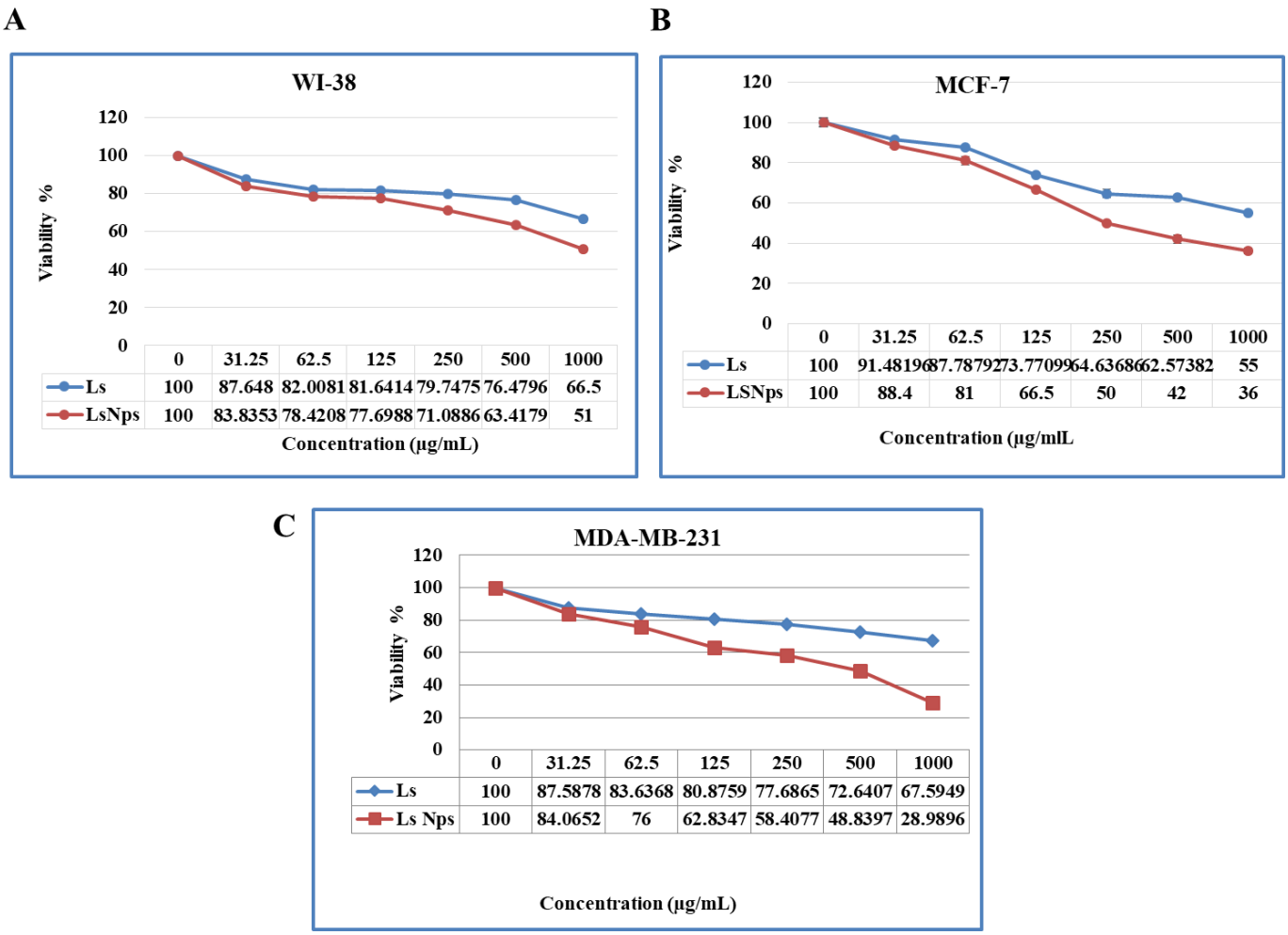

Figure 2 effect of Lepidium sativium (Ls) and Lepidium sativium nano particles (LsNps) against of A) WI-38, B) MCF-7 and C) MDA-MB-231 cells after 24 hrs of treatment with the indicated doses using MTT cell viability assay.

Detection of Apoptosis and necrosis

The three cell lines (WI-38, MCF-7, and MDA-MB-231) were treated with vehicle, 125, and 250 µg/ml of Ls Nps. In WI-38 cell line (fig. 3 A-C), microscopy images revealed less cell count after 24 hrs of treatment with LsNps250 µg/ml. Flow cytometry results (fig. 3 D-F) showed that there were 3 folds and 10 folds increase in necrotic cell death after treatment with 125 and 250 µg/mL Ls Nps respectively. There was also an increase in early apoptotic cell death (6.06 and 9.53 for 125 and 250 µg/ml Ls Nps respectively versus vehicle; 3.72).

Both breast cancer cell lines (MCF-7 and MDA-MB-231, fig.4 D-F, fig. 5 D-F) cell lines showed a shifting of the major population of the cells towards necrotic cell death with an increase in necrosis after adding a higher concentration of Ls Nps (125 and 250 µg/mL).

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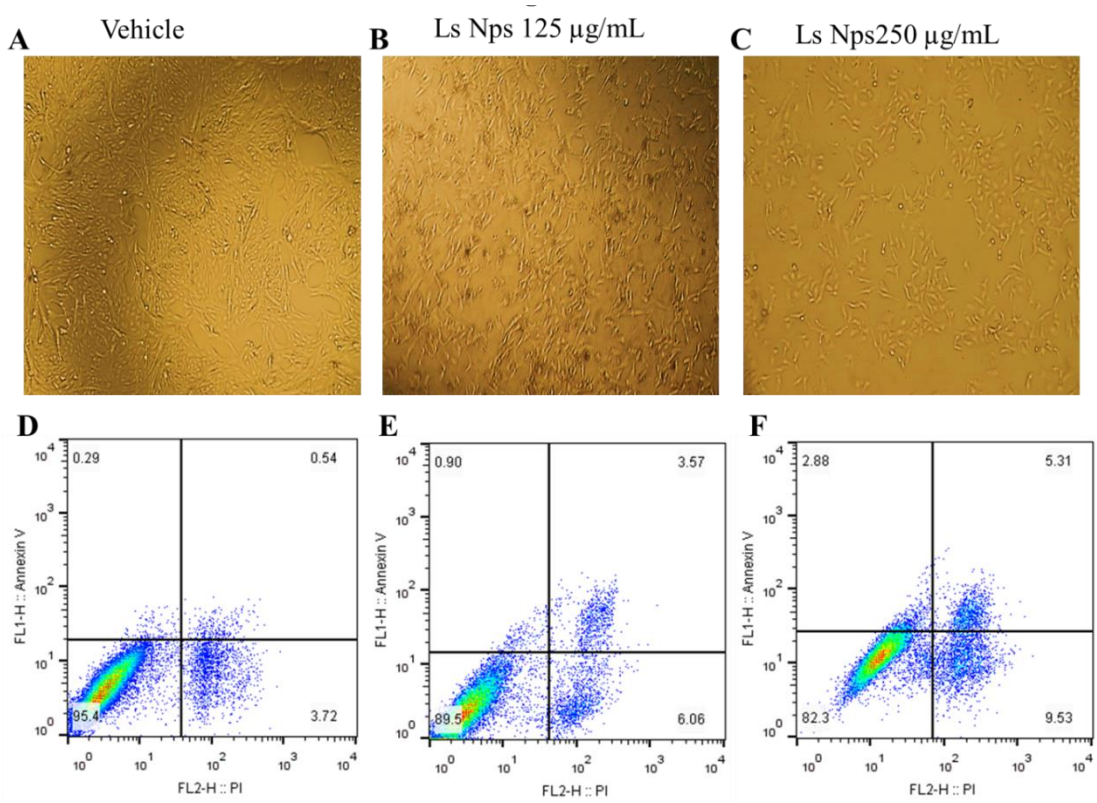

Figure 3 (A-C) bright field microscope images of WI-38 normal lung fibroblast cells treated with (Vehicle, 125 and 250 µg/ml of Ls Nps. Detection of Necrotic and apoptotic cell death using Annexin V and Probidium Iodid (PI) staining and analyzed using flow cytometry. (D) WI-38 vehicle treated cells, (E) WI-38 cell treated with 125 µg/ml of Ls Nps and (F) WI-38 cells treated with 250 µg/ml of Ls Nps after 24 Hrs of treatment.

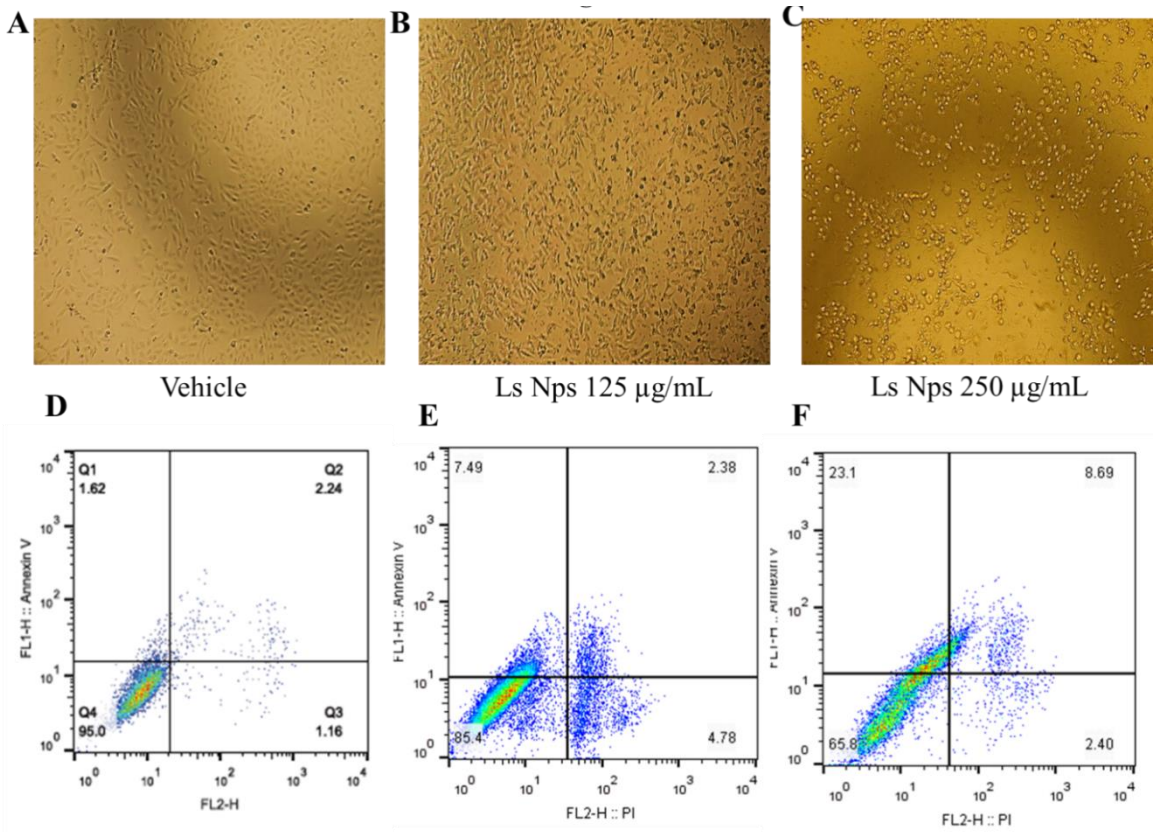

Figure 4 (A-C) bright field microscope images of MCF-7 breast cancer cells treated with (Vehicle, 125 and 250 µg/ml of Ls Nps. Detection of Necrotic and apoptotic cell death using Annexin V and Probidium Iodid (PI) staining and analyzed using flow cytometry. (D) MCF-7 vehicle treated cells, (E) MCF-7 cells treated with 125 µg/ml of Ls Nps and (F MCF-7 cells treated with 250 µg/ml of Ls Nps after 24 Hrs of treatment.

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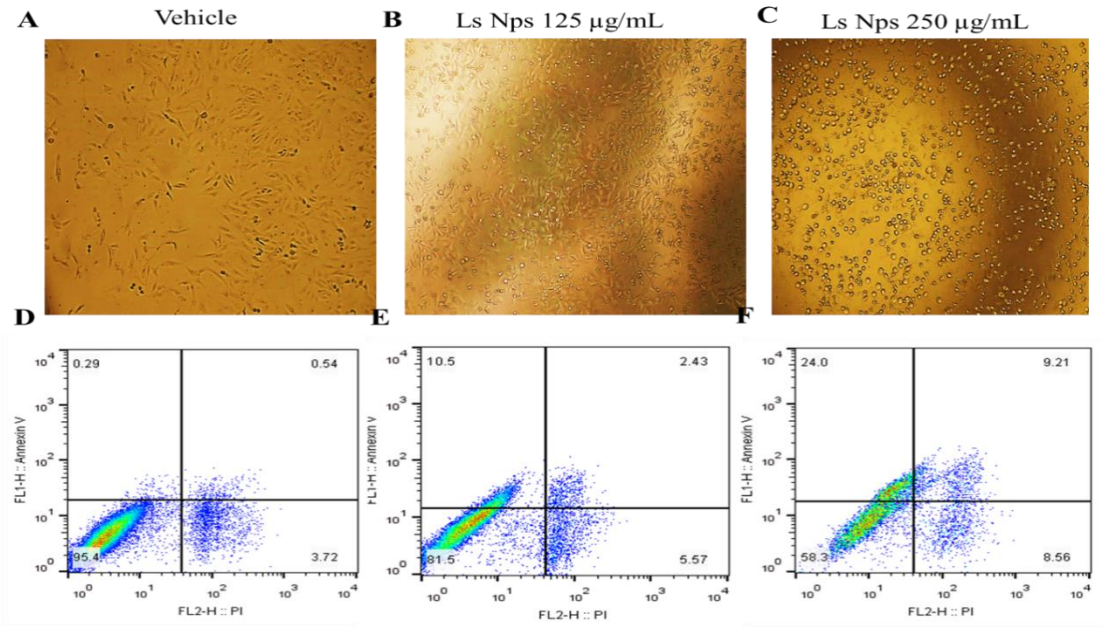

Figure 5 (A-C) bright field microscope images of MDA-MB-231 Breast cancer cells treated with (Vehicle, 125 and 250 µg/ml of Ls Nps. Detection of Necrotic and apoptotic cell death using Annexin V and Probidium Iodid (PI) staining was analyzed using flow cytometry. (D) MDA-MB-231 vehicle treated cells, (E) MDA-MB-231 cell treated with 125 µg/ml of Ls Nps and (F) MDA-MB-231 cells treated with 250 µg/ml of Ls Nps after 24 Hrs of treatment

Wound healing assay results

In the MCF-7 cell line (fig. 6), cell migration happened 24 hrs after treatment with vehicle or extract without being loaded in nanoparticles. However, cell migration was less visible when cells were treated with 125 µg/mL Ls Nps. First row is at 0 hr time point. Second row is 24 hrs after treatment. In MDA-MB-231 cells (fig. 7), the same pattern was observed with some inhibitionupon treatment with extract alone versus vehicle. The Ls Nps 125 µg/mL showed more inhibition of cell migration after 24 hrs period in MDA-MB-231 versus MCF-7.

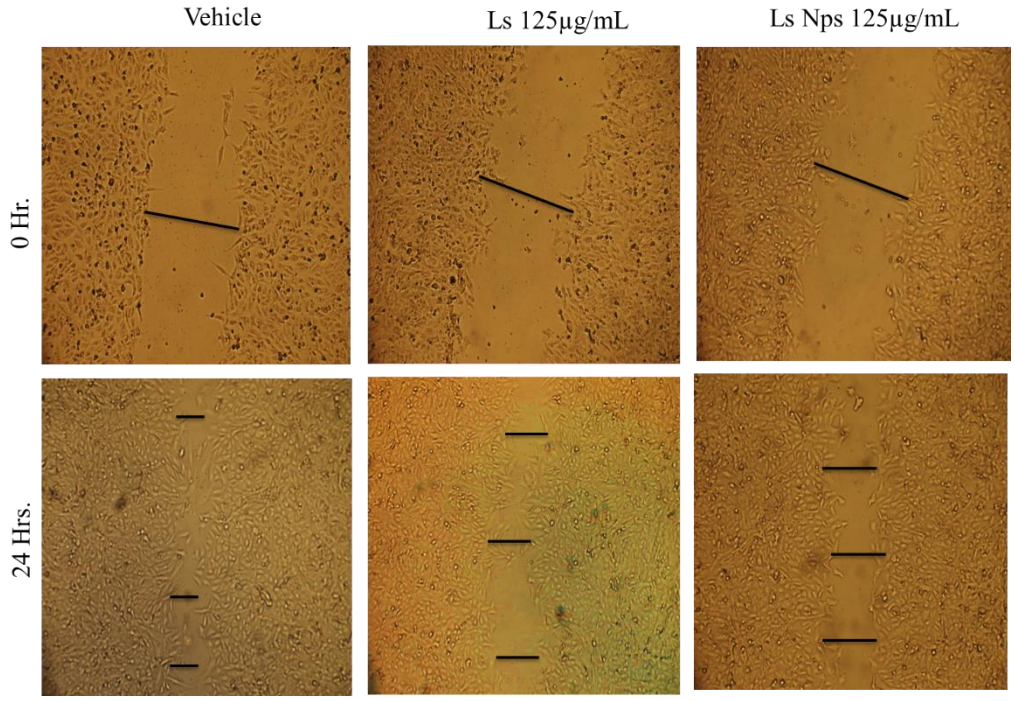

Figure 6 Wound healing assay and the effect of different preparations (vehicle, Ls extract, and Ls extract loaded into nanoparticles) on wound healing of the MCF-7 cells.

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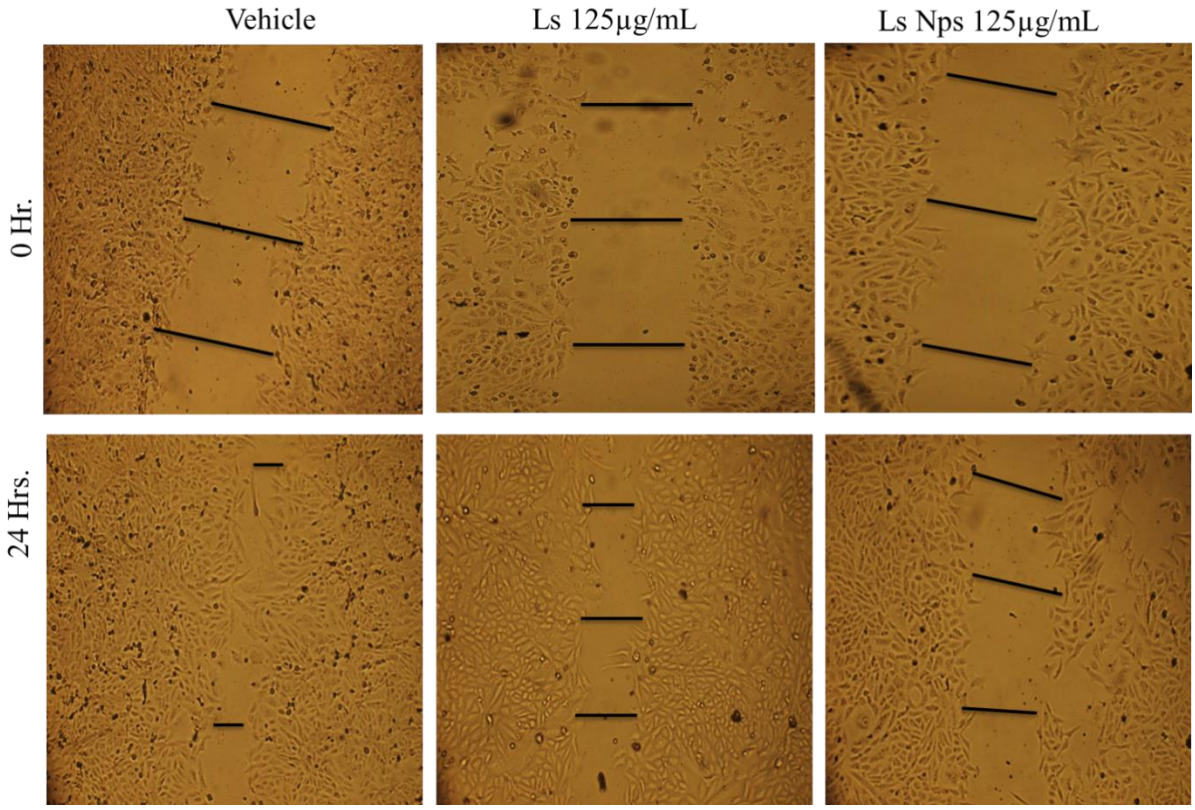

Figure 7 Wound healing assay. Effect of different preparations (vehicle, Ls extract, and Ls extract loaded into nanoparticles) on wound healing of the MDA-MB-231 cells. First row is at 0 hr time point. Second row is 24 hrs after treatment.

4. DISCUSSION

Lepidium sativum extract showed potential in the treatment of certain cancer types and other diseases or health issues. As demonstrated in the results section, there was a high entrapment efficiency of the Ls extract into the nanoparticles. This efficient loading of the extract molecules aided the delivery of the drug inside the MCF-7 and MDA-MB-231 cells. This is observed in the shifting of the general cells population from normal viable cells to death by necrosis and/or early apoptosis (figs. 3-5). Research on the health benefits of *Lepidium sativum* extract has been on the rise for the past few years. To our knowledge, this research was not published before. In the article of Basaiyye et al., (2019), studying the effect of *Lepidium sativum* extract on Jurkat E6-1 cells, the study showed that exposure to the extract caused early apoptotic death of the cells, observed in both flow cytometry assays as well as gene expression of apoptotic markers (BAX, FADD). On the other hand, the cell lines used here showed similar, but less pronounced effect, until loading the extract into nanoparticles and treating the cells with it, thus, validating the efficacy of the loaded nanoparticles in aiding the delivery of the extract and the targeting of the cells.

In Mahassni and Al-Reemi (2013), researchers test the effect of various concentrations (25, 50, and 75% concentration) of *Lepidium sativum* extract on MCF-7 cells. Only the concentration of 75% was able to cause necrosis in MCF-7 cells and other used cell lines, the other two concentrations were able to cause apoptosis in the cells, according to flow cytometry results. It is observed that the use of nanoparticles allows the use of a lower concentration of the extract while enhancing the targeting of the cells. In both breast cancer cell lines used in this article, wound healing assay results displayed almost normal proliferation after the addition of the vehicle alone, some inhibition of proliferation after the addition of the extract alone. The most effective inhibition was displayed upon treatment with Ls Nps at the concentration of 125µg/mL after 24 hrs treatment period. This provides an evidence for the efficacy of the nanoparticles formulation in enhancing the efficacy of the drug by optimizing its delivery to the cancerous cells. To our knowledge, no previous studies have published similar results or used the same methods of loading the *Lepidium sativum* extract onto nanoparticles.

5. CONCLUSION

In our opinion, more research is needed to test the efficacy of the nanoparticle formulation *in vivo*, to assess its potential for future therapeutic uses, and whether it could enhance the wellbeing of breast cancer patients by reducing the side effects. In conclusion,

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Lepidium sativum extract exerts some efficacy alone against the used breast cancer cells. The use of nanoparticles to deliver the drugs enhances its efficacy and targeting.

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Conflict of Interest

The authors declare that there are no conflicts of interests.

Author Contributions

All parts of the research were done side by side between the authors.

Data and materials availability

All data associated with this study are present in the paper.

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